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A System for Investigation of Biological Effects of Diagnostic Ultrasound on Development of Zebrafish Embryos

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Abstract

A system for scanning zebrafish embryos with diagnostic ultrasound was developed for research into possible biological effects during development. Two troughs for holding embryos were formed from agarose in a rectangular dish and separated by an ultrasound absorber. A 4.9 MHz linear array ultrasound probe was positioned to uniformly scan all the embryos at the bottom of one trough, with the other used for controls. Zebrafish embryos were scanned continuously from 10–24 h post fertilization (hpf) during the segmentation period and gross morphological parameters were measured at 30 hpf, including viability, length, number of visible axons, and the progression of the lateral line primordium (LLP). Our initial tests were encumbered by the thermal effects of probe self-heating, which resulted in accelerated development of the zebrafish embryos. After subsequent optimization, our test revealed a significant retardation of primary motor axons and the migration of the LLP in embryos scanned with ultrasound, which indicated a potential for nonthermal effects on neuronal development. This diagnostic ultrasound exposure system is suitable for further investigation of possible subtle bioeffects, such as perturbation of neuronal migration.

Introduction

The development of medical diagnostic ultrasound imaging 50 years ago engendered interest in any possible adverse biological effects. Ultrasound imaging was considered safe with regard to stochastic effects of the kind seen with ionizing radiation, and therefore, potentially ideal for imaging in obstetrics and pediatrics. However, little was known about the interaction of diagnostic ultrasound with biological systems. Therapeutic ultrasound was well known to cause dangerous effects through the mechanisms of heating and ultrasonic cavitation. Subsequent research on potential bioeffects of diagnostic ultrasound on the embryo and fetus may be considered meager relative to the large potential collective impact presented by the nearly universal prenatal exposure of humans *in utero*, for which even relatively rare events may be important for public health. Authoritative reviews of the problem have found that obstetrical ultrasound as currently practiced appears to be relatively safe from effects of heating or cavitation, but have lamented the lack of more and better information on possible risks of subtle bioeffects for different ultrasound modes, examinations and output exposure levels.[1–4]

Ultrasound imaging methods continue to evolve. For example, ultrasound investigation of even the first trimester embryo or fetus is now common for gestational ages as early as the 5th week.[5] Such early examinations can be difficult and involve lengthy applications of imaging and Doppler modes.[6] The observation of nuchal translucency at 11–14 weeks of pregnancy recently has become a textbook screening method for possible abnormalities using transabdominal or transvaginal two-dimensional imaging.[7,8] Two-dimensional and spectral Doppler ultrasound are used in detecting cardiac abnormalities in the first trimester as a new prenatal screening tool.[9–11] These recent developments have elevated the level of interest in possible developmental bioeffects of diagnostic ultrasound.[12]

Although research on bioeffects of diagnostic ultrasound has been minimal in recent years, studies are published occasionally which appear to detect unsuspected bioeffects using new biological methods and modern diagnostic ultrasound machines. For example, Ang *et al.* showed that diagnostic ultrasound exposure of pregnant rats impacted the migration of neurons and created concern about possible subtle learning or behavioral consequences in humans.[13] Scanning was at 7.5 MHz for periods up to 7 h. This study

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generated fear of a link between diagnostic ultrasound and autism, but little scientific information exists to appraise this fear.[14] Another recent study found that apoptosis of liver cells could be induced by 5.8 MHz Doppler mode imaging of rat fetuses *in utero* for up to 10 min, which also might be of concern.[15] More research is needed using appropriate animal models, state of the art research methods and modern diagnostic ultrasound machines to explore such possibilities and continually test the assumptions of safety for diagnostic imaging of the fetus.

Thorough studies of developmental bioeffects in rats or larger animal models of human diagnostic imaging are very complex, time consuming and expensive. Basic information can be derived from *in vitro* cellular studies; for example, ultrasound appears to be capable of modulating neuronal development.[16] However, translation of *in vitro* results to *in vivo* conditions is problematical. A smaller animal model, which has been proven in tests of developmental impacts of a variety of insults, would be of value for rapid but in-depth studies of various possible bioeffects of ultrasound in obstetrics. The Zebrafish (*Danio rerio*) appears to be an excellent candidate.[17] Advantages of this animal model are its small size, the transparency of the embryos, the ability to generate large numbers of embryos at minimal expense and *ex utero* embryonic development, which allow following the entire development from fertilization to nearly full development in a few days. This animal has been used in numerous studies of developmental or environmental toxicology.[18,19] In addition, the Zebrafish embryo has been shown to be adversely effected by various physical insults, including heat and vibration.[20,21] The transparency of the embryos and availability of specific antibody staining allows the detailed investigation of axonal and neuronal development,[22,23] and may provide a means to study complex human conditions, such as autism.[24]

The goal of this study was to create reliable diagnostic ultrasound exposure methods for zebrafish embryos and to test them in preliminary experiments. Various approaches were tried and the final design allows for simultaneous scanning and sham scanning of groups of embryos for extended periods. Long exposures are useful as a screening tool for effects at a range of time points without the need for numerous brief tests at each stage of development. Finally, the system was used for preliminary tests to verify its utility by scanning during the segmentation stage and examining gross morphological parameters for ultrasound-related differences. The system should serve well for investigations of bioeffects of diagnostic ultrasound on neuronal migration, gene expression and other possible subtle changes.

Methods

Animal preparation

Fertilized zebrafish embryos were obtained from the Department of Pediatrics and Communicable Diseases breeding facility. The wild type breeders (AB*) are maintained according to the protocol approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan. These were transferred to zebrafish medium with methylene blue and incubated at 28.5°C. At 9 h post fertilization, 32 embryos were selected for viability and an appearance indicative of the late gastrula period of development.[25] These were randomly divided into two equal groups for ultrasound scanning and sham scanning.

Ultrasound

The ultrasound scanning was provided by a diagnostic ultrasound system (Z.One Ultra; Zonare Medical Systems, Mt. View, CA) with L14-5w linear array probe. The probe was operated in the harmonic B-mode designated H12MHz at 15 frames per second. The Mechanical Index, an on-screen exposure metric for nonthermal mechanisms, was set to its maximum value of 1.5. The Thermal Index, an on-screen exposure metric for the thermal mechanism, was 0.1. The image was about 5.6 cm wide and had a depth setting of 4 cm. The ultrasound field was characterized in a water bath using a calibrated hydrophone (model 805; Sonora Medical Systems, Inc., Longmont, CO). The maximum pulse at 1.5 cm depth was measured on an oscilloscope (TDS 520B; Tektronix, Inc., Beaverton, OR), as shown in Figure 1. This pulse had peak positive and negative pressure amplitudes of 7.3 and −3.6 MPa, respectively, and a duration of 464 ns. The peak negative pressure amplitude corresponds to a Mechanical Index of 1.6, which is well within the upper limit of 1.9 for

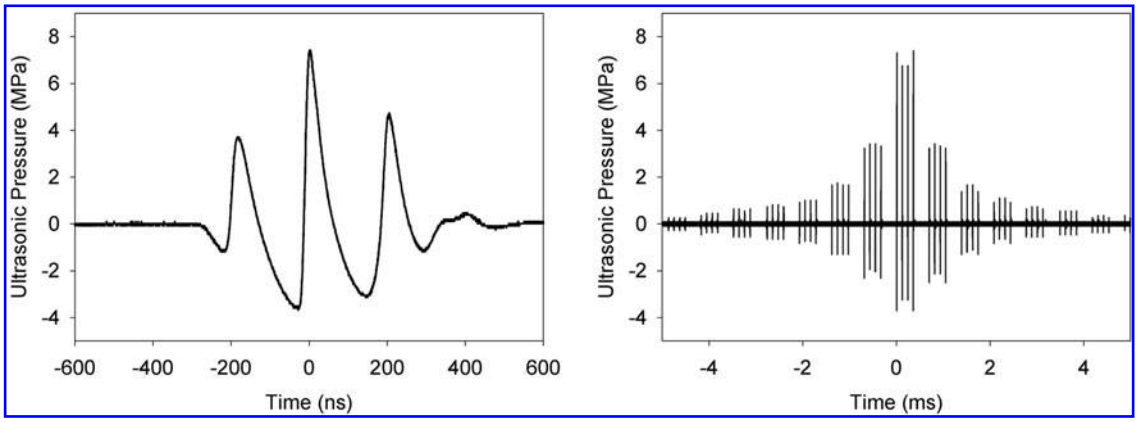

FIG. 1. Plots of the ultrasonic pressure delivered to a point during scanning for the maximal pulse (*left*) and for the sequence of pulses for one image (*right*). The deviation from a sinusoidal waveform is due to nonlinear propagation. In the figure on the *left*, the inter-pulse spacing is 120 μs within sets of four pulses arriving at 700 μs intervals.

diagnostic ultrasound. The peak-to-peak duration of one cycle was 205 ns, which indicates a center frequency of about 4.9 MHz. The calculated spatial-peak, pulse-average intensity was 566 W/cm^2. The pulse repetition frequency was 8.5 kHz, with an image pulse sequence (the series of pulses of increasing and decreasing amplitudes recorded as the scanning beam passed by the hydrophone) as shown in Figure 1. The thickness of the scanned beam was comparable to the size of the embryos, as shown in Figure 2, which allowed whole-embryo exposure for carefully aimed scanning. The normalized peak rarefactional pressure amplitude was measured across the scan plane at four positions along the trough location, and the mean values are plotted in Figure 2. The half-pressure (−6 dB) and half power (−3 dB) beam widths were 2.2 and 1.5 mm, respectively. The chorion and yolk were 1.3 and 0.7 mm in diameter, respectively. The length (−6 dB) of the scanned focal zone was about 2.3 cm, centered on the trough, and the scanned width was 5 cm.

The exposure system consisted of a plastic board with the probe mounted on one side and an 8.5 cm wide by 10 cm long plastic dish on the other side. The 3.5 cm deep dish was fabricated from a cell culture flask (75 cm^2). The board was recessed to accept the dish and hold it in position for transmission of the ultrasound through a thin-film acoustical window into the dish. For setup, the dish was filled to a depth of 2.5 cm with 3% agarose (A-2790; Sigma Aldrich Co., St. Louis, MO) prepared in zebrafish medium. Two V-shaped plastic pieces were set to create two troughs aligned in parallel with the face of the probe in the agar. The rationale for this design was that it allowed accurate positioning of a row of embryos at the focal depth of the probe, with little or no attenuation or reflections of the ultrasound. The two troughs allowed simultaneous sham and scan exposure with essentially identical culture conditions. A 2 cm thick slab of ultrasound absorbing rubber was also included between the troughs, so that only one trough was exposed to the ultrasound. This arrangement is shown from above in Figure 3. After the agarose hardened, the plastic molds were removed; the dish was filled with zebrafish medium, and then placed in the incubator for temperature equilibration.

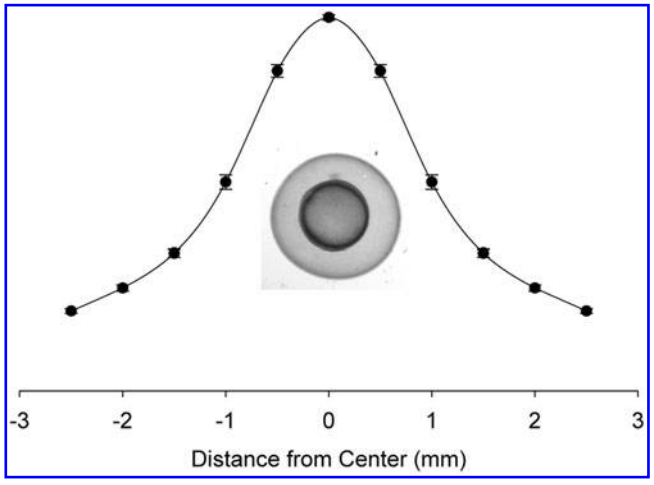

FIG. 2. The width profile of the ultrasound beam was comparable to the size of an embryo (*inset*), which allowed for whole-embryo exposure with image guided aiming of the probe.

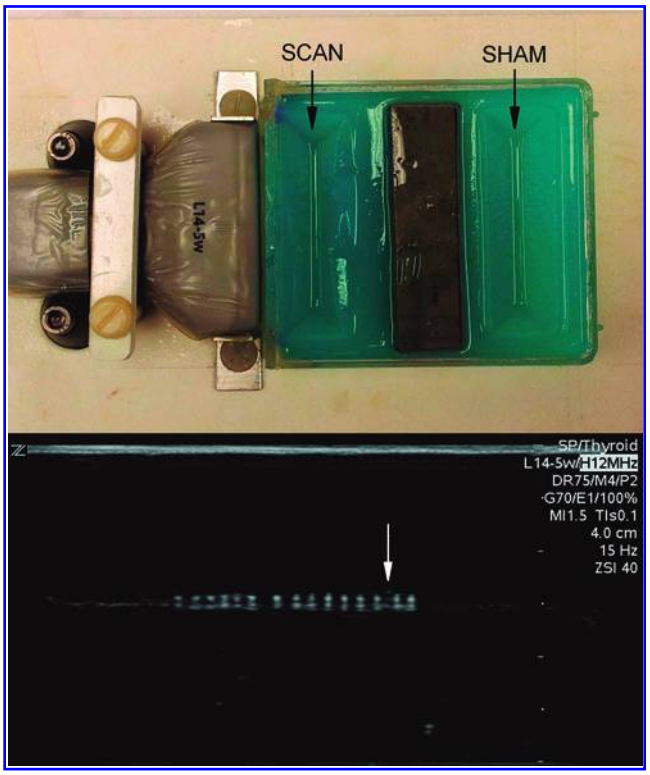

FIG. 3. *Top*: The exposure apparatus photographed from above. The L14-5w probe is on the *left* and aimed into the dish. The scan and sham troughs were formed in agar parallel to the probe face, and were filled with zebrafish medium during testing. A slab of ultrasound absorbing rubber is positioned between the troughs to eliminate exposure of the sham trough. The rectangular dish was 8.5 cm wide by 10 cm long. *Bottom*: An ultrasound image guidance at the start of run 1 with the probe face directed downward from the top of the image. The bottom of the scan trough can be seen as a line of weak echoes at about 1.5 cm. Clustered at the middle of the trough are the 16 zebrafish embryos aligned in the trough. Each embryo appears as a bright double-echo, one from the front of the chorion and one from the back. One embryo (*arrow*) was slightly out of place in this image. Color images available online at www.liebertpub.com/zeb

Zebrafish embryos can be obtained in large numbers, allowing rapid collection of data of high statistical quality. However, this strategy requires uniform scan conditions for all embryos. In our system, this was accomplished simply by aiming the probe to obtain the brightest image of the embryos in the trough, with uniform brightness for embryos across the field of view. For exposure, the selected embryos were randomly placed in the scan or the sham scan trough. The probe mount was then adjusted to obtain an image of the embryos to be scanned at 1.5 cm depth, which gave the brightest embryo echoes, as shown in Figure 3. The final adjustment using the ultrasound image assures the uniform simultaneous exposure of all the embryos at the same acoustical field level. Without the image guided aiming, the scanning beam might not hit each embryo directly or even miss some embryos. In Figure 3, one embryo was slightly out of the scan plane, which was corrected by gentle shaking of the dish. Finally, the entire assembly was placed in the incubator. As described below, the assembly was either surrounded by air with ultrasound

coupling medium between the probe and window, or by water in a shallow pan. In the latter case, the water served as coupling medium and as a thermal buffer for probe heating.

Exposure was begun at ~10 h postfertilization (hpf) and continued overnight until 24 hpf, when the embryos were removed from the dish and placed into 35 mm Petri dishes filled with zebrafish medium. This 14 h exposure period constituted approximately the entire segmentation period of development. At this point, the embryos would normally begin to make periodic motions, which were noticeable in the ultrasound image but scored under a stereomicroscope. The embryos were cultured for an additional 5 hr before scoring to allow any delayed effects to emerge, for example, disturbances in somite formation can take several hours to appear in the somite pattern.[20]

Measured endpoints

At ~29 hpf, the embryos were scored for viability by observing embryo motion. The chorions were then removed with the aid of a 2 mg/mL pronase (Roche Diagnostics, Indianapolis, IN) solution in zebrafish medium. The embryos were fixed overnight at 4°C in BT fixative and processed for staining (page 8.8 in Ref.[17]) except that fluorescent antibody stains were used. The primary antibody was ZNP-1(Zebrafish International Resource Center, University of Oregon, Eugene, OR) which targets rhombomeres, including primary motor axons, and the secondary label was goat anti-mouse IgG labeled with Alexa Fluor 488 (Life Technologies, Carlsbad, CA). The lengths of the embryos were measured by a straight line (i.e., not accounting for tail curvature), and somite formation was checked in brightfield microscopy. The axons were visualized in green fluorescence for gross morphology and the total number of visible axons was counted. The somite and axon development can be affected, for example, by heat shock.[20] In addition, the lateral line primordia (LLP) were observed with differential interference contrast (Leica DMRB; Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) and scored for position relative to the axons. The LLP migrate during the pharyngula period of development, a process which can be perturbed by variation of chemokine signaling.[26,27] Figure 4 shows an example of the zebrafish

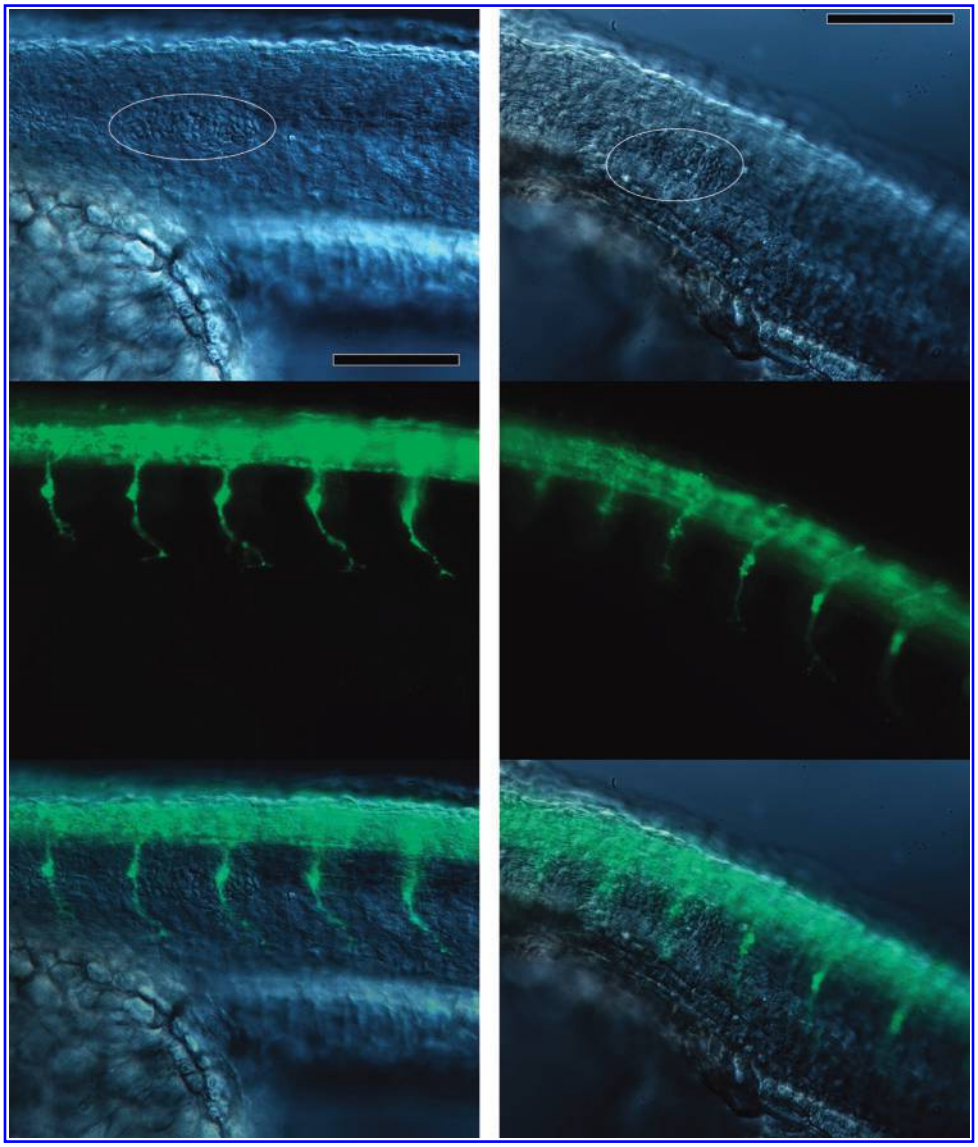

FIG. 4. Microscope images for a sham (*left*) and a scanned embryo (*right*). *Top*: Differential interference contrast images with 40× objective showing the middle of the zebrafish body after sham exposure in experiment 4, with the yolk and yolk extension at the bottom of the image. The lateral line primordium (LLP) is seen as a closely packed group of cells within the ellipse. The scale bar represents 100 μm. *Middle*: Fluorescence images of the stained primary motor axons, which extend from the spinal cord, showing axons 5–9 and 4–9 for the sham and scanned embryos, respectively. *Bottom*: Merged images showing the position of the LLP with the leading edge between axon 7 and 8 (scored as 7.5) and between axon 6 and 7 (scored as 6.5) for the sham and scanned embryos, respectively. The somite borders, which run roughly parallel to, and midway between the axons were not seen clearly at this focal level. Color images available online at www.liebertpub.com/zeb

microscopy with the LLP identification, the fluorescence image of primary motor axons, and the combined image showing the position of the LLP relative to the axons. For scoring, a lower magnification was used to allow counting of the axons from anterior to posterior positions. These preliminary measured endpoints were selected as simple but important morphological features of the embryonic development, to demonstrate the utility of the experimental setup with regard to survival and gross development (rather than on the basis of an expectation that any specific biological effect should occur). Gross teratological effects are not expected from diagnostic ultrasound and the system must be sufficiently refined to reveal potentially important, but subtle, bioeffects.

Results

After preliminary work to develop the system described above, a series of embryo experiments was begun to evaluate the system. Four such experiments were made with mixed results and an evolution in the methods. In the first experiment, the exposure apparatus was incubated in air. All embryos survived and were moving at the end of the exposure; however, one scan and two sham embryos had rumpled chorions. Only 10 of each group were scored due to damage of embryos during dechorionation, processing and handling (e.g. yolk reduction) for microscopy. There were no clearly evident deviations of the morphology of the scanned embryos relative to the shams. Results are listed in Table 1 for the three measured endpoints. Subsequent testing with additional embryos indicated that the rumpling of the chorion exhibited by some embryos could be eliminated by preparing the agarose in the dish at least 12 h in advance of use in an experiment. In the subsequent experiments, no rumpling was seen.

In the second experiment, the apparatus was again incubated in air. All embryos survived and were moving at the end of the exposure. No obvious deviations in morphology were seen. Ten randomly selected embryos were scored from each group. For this experiment, the sizes of the sham embryos were significantly larger than the scanned embryos, but the number of axons and the position of the LLP were both significantly larger for the scanned embryos, see Table 1. This interesting finding led to a search for systematic differences in temperature between the two groups during scanning. Heating from the absorption of ultrasound was not expected due to the low Thermal Index of 0.1 for the pulsed and scanned exposure. However, the probe itself was found to be warm when operated in air, and reached a maximum of about 3°C above room temperature after 2 h. Using a differential thermometry system (BAT-10, Physitemp Instruments, Inc., Clifton, NJ), a constant elevation of 1.4°C was found after 6 h at the scan trough relative to the sham trough. Subsequently, the apparatus was incubated in a pan of water (rather than air), which provided thermal stabilization and water coupling of the ultrasound probe to the dish through a $\sim$1 mm gap to reduce conduction of probe heat into the dish. This modification reduced the temperature differential to less than 0.4°C.

For the third experiment, only a small number of embryos were available, so that the selection process was very limited. One scan embryo was not viable after the scanning period. Five of each group, which had unusually small chorion diameters, showed clearly restricted development and were excluded from scoring. Ten of each group survived and were moving after the scanning period. The measurement results were highly variable, with no significant differences, see Table 1.

For the final experiment, the supply of embryos was restored and 16 excellent embryos were selected for each group. After scanning, all the embryos survived and were moving. All 16 in each group were scored and no gross deviations of morphology, including the appearance the eye, brain and primary motor axons, were noted. The mean sizes of the embryos were not significantly different, see Table 1. However, the number of visibly developed primary motor axons and the position of the LLP were both significantly less for the scanned group. This was a marked change from the results of experiments 1 and 2 with the apparatus incubated in air. In Figure 4, photomicrographs are shown for a sham and a scanned embryo, which illustrates the different in LLP progress. The results for the axon numbers and LLP position for all experiments are plotted in Figures 5 and 6, respectively. The difference between the first two experiments incubated in air with advanced positions of the LLP in the scanned groups is noteworthy relative to the last two experiments incubated in the water pan with retarded positions of the LLP.

Discussion

A system for scanning zebrafish embryos with diagnostic ultrasound was developed and tested for use in research into possible biological effects of ultrasound during development. The system included a dish with two troughs formed in agarose, one for scanned and one for sham-scanned embryos, which were separated by an ultrasound absorber. This allowed culture conditions of the sham and scanned groups to be essentially identical. The troughs were tested with 10–16 embryos, but could contain 30–40 embryos for each experiment. A 4.9 MHz linear array ultrasound probe was positioned with guidance from the image to scan all the embryos at the bottom of the trough to give uniform ultrasound exposure with minimal perturbation of the free-field acoustical conditions. The exposure system with embryos was located in

Table 1. The Results for the Three Measured Endpoints for Each Experiment (Exp)

		Embryo size (mm)			*Number of axons*			*LLP position*		
Exp	*N*	*Sham*	*Scan*	*p*	*Sham*	*Scan*	*p*	*Sham*	*Scan*	*p*
1	10	1.7 ± 0.2	1.7 ± 0.1	NS	22 ± 2.8	23 ± 1.8	NS	5.9 ± 1.2	6.7 ± 1.1	NS
2	10	2.2 ± 0.2	2.0 ± 0.2	< 0.05	23 ± 1.6	27 ± 1.0	< 0.001	8.3 ± 2.2	11.7 ± 1.8	< 0.001
3	10	2.0 ± 0.4	1.7 ± 0.2	NS	23 ± 3.9	20 ± 2.6	NS	6.8 ± 3.8	4.2 ± 2.0	NS
4	16	2.3 ± 0.3	2.2 ± 0.3	NS	25 ± 1.0	22 ± 1.8	< 0.001	7.1 ± 0.8	5.5 ± 1.6	< 0.002

Values are given as the mean plus/minus the standard deviation with no significant difference (NS) for $p > 0.05$ using the Student's t test.

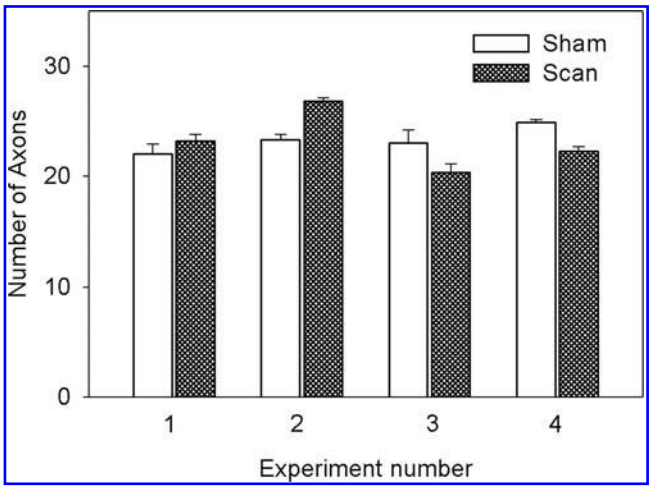

FIG. 5. Results for the number of axons, which develop near the middle of each somite. The scan result was significantly larger than the sham for experiment 2 ($p < 0.001$), but significantly smaller for experiment 4 ($p < 0.001$). The differences seen in experiments 1 and 3 had similar but not significant trends.

an incubator operated at 28.5°C during testing. Scanning was continued from 10–24 hpf during the segmentation period of development. Gross morphological parameters were measured at 30 hpf, including length, number of visible primary motor axons, and the progression of the LLP relative to the somites (counted relative to the axons).

In four experiments, a variety of problems were encountered and corrected to improve the utility and consistency of the tests. An agarose curing period of at least 12 h was found to be needed to avoid rumpling of the chorions. Testing after the second experiment revealed a small temperature elevation due to self-heating of the probe, which was corrected by placing the system in a water pan for heat dissipation and temperature stability. The third experiment was confounded by a shortage of embryos, but the final preliminary experiment confirmed the utility of the system for long-term exposure. Variation in results were generally small, but could be improved by narrower staging for the groups and better handling of the embryos during evaluation. Statistically significant differences between the scan and sham groups were found in the second and fourth experiments, which had minimal problems with obtaining or handling the embryos.

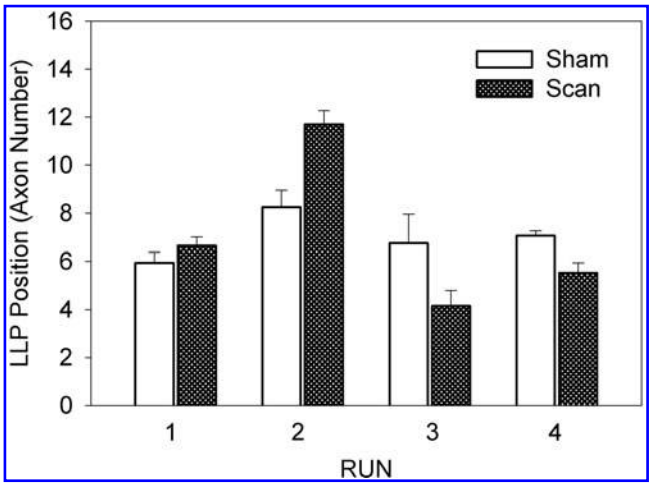

FIG. 6. Results for the position of the LLP relative to the axons. The scan result was significantly larger than the sham for experiment 2 ($p < 0.001$), but significantly smaller for experiment 4 ($p < 0.002$). The differences seen in experiments 1 and 3 had similar but not significant trends.

For the second experiment, the mean sizes were slightly less for the scanned embryos, but the number of axons and the position of the LLP were significantly advanced in the scanned group, see Table 1. A temperature elevation of 1.4°C was detected in the scan trough from the warm probe, which was likely involved in the observed developmental differences for the first two experiments. Zebrafish embryo development is known to proceed normally over a 25°C–33°C temperature range, with heat shock effects requiring temperatures of 39°C–40°C for 30 min.[20] For small temperature elevation, the development is accelerated in accord with a linear formula.[28] An elevation to 29.9°C for 14 h would be expected to show about 1 h faster progression relative to 28.5°C incubation, which plausibly explains the significant differences in axon and LLP development found in experiment 2. The temperature elevation was produced by self-heating of the probe, rather than by ultrasound absorption-heating, which would not be expected to be a factor for human patients. Therefore, the temperature elevation was substantially reduced for subsequent experiments by incubating the system in a water pan.

The final experiment, which had the fewest technical problems, resulted in a significant reduction in axon number and of LLP migration in the scanned group, see Figures 5 and 6. These important results suggest a potential for the induction of nonthermal bioeffects of diagnostic ultrasound on the embryo development. It should be noted that this experiment did not address whether or not these bioeffects might be adverse, or persistent into the adult stages. More research would be needed to clarify these uncertainties and to test for subtle neuronal bioeffects, which might be harmful.

The system described here could be used for the investigation of various possible subtle bioeffects, such as the perturbation of neuronal migration reported in mice.[13] Several neuronal migration events have been well characterized in zebrafish.[29–32] Using the system described here, large numbers of embryos could be studied relatively quickly, allowing for testing a range of different events and exposure parameters. Long duration scanning can easily be performed (e.g. 14 h in this study) to simulate the longer duration exposures of Ang *et al.*[13] Alternatively, specific migration events could be selected and followed for shorter periods by taking advantage of the various labeling and observation methods available with these transparent organisms. Modifications of the system could readily include placement of individual embryos to provide consistent orientation relative to the ultrasound beam direction and the use of older embryos or larvae under anesthesia to study later developmental events. Other endpoints could be readily included in research, such as analysis of gene expression. Extended studies, such as observation of behavioral parameters in adults, could be accomplished with zebrafish much faster and more economically than with mice. Overall, the zebrafish developmental model combined with this novel exposure system could rapidly screen for a variety of subtle developmental effects, and fill the need for modern

bioeffects information to provide assurance of the safety of obstetrical ultrasonography.

Acknowledgments

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Disclosure Statement

No competing financial interests exist for this study.

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